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Epidermal growth factor (EGF) family of ligands bind to a number of related receptors and stimulate mitogenesis in mammary cells. Signal transduction downstream of the EGF family of receptors involves activation of the GTPase Ras and its effectors.*RSU-1*, a cDNA identified as an inhibitor of Ras-induced transformation, was HA-epitope tagged and introduced into expression vectors. The HA-*RSU*-1 vectors were transfected into MCF7 and MCF10A cell lines. Transfectants were selected and tested for response to EGF and serum stimulation. Analysis of the several kinase pathways which have been characterized as dependent on Ras activation were tested in the *RSU*-1transfectants following stimulation of serum starved cells with EGF or serum. Expression of HA-Rsu-1 resulted in resulted in an increase in activation of Erk-2 kinase and a decrease in activation of p54 Jun kinase. Activation of AKT kinase by serum was unaffected by Rsu-1 expression. These results demonstrate that Rsu-1 can alter signal transduction downstream of Ras in MCF7 cell line. HA-Rsu-1 transfectants had similar anchorage dependent growth rates as the control cell lines, but exhibited a reduction in anchorage independent growth.

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INTRODUCTION

Epidermal growth factor (EGF) family of ligands bind to a number of related receptors and stimulate mitogenesis in mammary cells. Signal transduction downstream of the EGF family of receptors involves activation of the GTPase Ras and its effectors. Expression cloning to isolate inhibitors of Ras-induced transformation identified the Rsu-1 cDNA [4]. Previous experiments demonstrated that the expression of the Rsu-1 gene in NIH3T3 fibroblasts under the control of a heterologous promoter could suppress transformation by v-Ras but not v-Raf, v-Mos, or v-Src oncogenes [4]. Experiments in our laboratory have determined that the Rsu-1 suppressor inhibits EGF- and TGFα- induced anchorage independent growth of NOG8 mouse mammary cells. *rsu-1* is a highly conserved single copy gene which encodes a protein of 33kD. p33 Rsu-1 contains a series of 23 amino acid leucine-based amphipathic repeats homologous to the repeats found in yeast adenylyl cyclase in the region through which Ras activates adenylyl cyclase in *Sa. cerevisiae* [4]. This suggested that Rsu-1 might interact with Ras or Ras-regulatory or -effector molecules. Transfection of a Rsu-1 vector into NIH3T3 cells and PC12 cells resulted in an increase in signal to Raf-1 and Erk-2 kinase and a decrease in signal transduction to Jun kinase [8]. These results demonstrate that Rsu-1 can alter signal transduction downstream of Ras.

Signal transduction "downstream" of Ras depends on the association of Ras with its effector proteins. Several proteins have been identified which associate with Ras in a GTP-dependent manner. These include Raf-1, RasGAP, p110 subunit of PI-3-kinase, Rin-1, Mek kinase 1, protein kinase C zeta, and RalGDS; in the case of several of these proteins (i.e. Raf-1, RasGAP, p110 PI3kinase) signal transduction pathways activated as a result of interaction with Ras have been characterized. Activation of Ras effectors can lead to phosphorylation and activation of kinases, cytoskeletal proteins and transcription factors. For example, activation of Raf-1 results in specific phosphorylation of Mek and Erk-2 [14], and use of dominant negative Erk demonstrated that inhibition of this pathway interefered with Ras transformation in fibroblasts [3]. The small G proteins Rac and Rho are activated independently of Raf-1 by an as yet uncharacterized mechanism in response to activated Ras, and very recent evidence suggests that they play a crucial role in growth and tumorigenesis of epithelial cells. Studies using dominant negative Rho and Rac indicated that inhibition of Rho and/or Rac pathways prevented transformation by Ras, suggesting that these proteins regulate pathways essential for Ras transformation [6, 12, 13]. Activated Rac and cdc42 induced transformation in epithelial cells [7], whereas activation of the Raf-1/Erk pathway alone was not sufficient to transform rat intestinal and MCF10A epithelial cell lines [10]. These results, along with studies describing properties of transformants produced by specific effector mutants of Ras [1, 7, 15], have led to the conclusion that several pathways "downstream" of Ras contribute to transformation of cells in response to activated Ras. Most interestingly, activation of Rho and Rac appears to be responsible for induction of anchorage independent growth and tumorigenicity in epithelial cells [7]. Because cdc42Hs and Rac have been shown to be required for the activation of the stress-activated or Jun kinase [2, 9, 11], and RhoA has been shown to be necessary for activation of the SRF by serum and other agents that act through the pertussis-sensitive G protein pathway [5], it appears that the small G proteins may exert their effects via transcriptional activation. Because pathways dependent on Rac and Rho are so important for maintaining the transformed state, inhibitors of this pathway should prove especially useful in further dissection of growth control pathways and in developing antitumor therapies. Because the Rsu-1 suppressor has been shown to inhibit transformation and events dependent of Rac and Rho [8] we are investigating its role in the growth regulation of breast carcinoma.

Current experiments are designed to assess the ability of Rsu-1 to regulate pathways "downstream" of Ras which are activated by members of the EGF receptor family in human mammary carcinoma cells. Rsu-1 transfectants have been prepared in the MCF 7 cell line and are being isolated from the MCF 10A cell line for analysis of response to EGF and estrogen. Rsu-1 transfectants will also be prepared

in MDA-MB468 cell line. The Rsu-1 transfectants are being compared to vector control transfectants for anchorage-dependent and -independent growth in response to growth factor and estrogen. MCF7 transfectants will be compared to vector control transfectants for their ability to form tumors in athymic mice. The effect of Rsu-1 on the Ras-induced activation of downstream effectors (GAP, Raf-1, Mek kinase, and PI3-kinase) is being determined by analyzing the kinases regulated by specific Ras effectors following stimulation of the cells. The kinases include: MAP kinases Erk-2, Jun kinase, p38 kinase, and Akt kinase (protein kinase B).

BODY:

Several vectors have been used for the expression of p33Rsu-1 in human breast cancer cell lines. These vectors include: p3v36 which contains the *RSU-1* cDNA under the control of a MT-1 promoter in a retroviral vector [4], p3v65 which contains an HA-tagged version of the *RSU-1* cDNA under the control of a RSV promoter in a vector containing an SV40 origen of replication [8], and p3v64 which contains an HA-tagged *RSU-1* cDNA in a retroviral vector under the control of a MT-1 promoter. MCF 7 and MCF10A cell lines have been transfected or infected with the above vectors and the appropriate "empty" control vectors. Following selection in G418 single colonies have been isolated using cloning cylinders, expanded into cell lines and screened for *RSU-1* RNA expression. In the case of transfectants contining the p3v36 or p3v64 vectors the transfectants were assayed in the presence and absence of Cd++ which results in induction of transcription from the MT-1 promoter. The following table lists transfectants prepared to date.

MCF 7	p1521	control vector	transfectants obtained
MCF7	p3v36	*RSU-1* vector	transfectants obtained
MCF7	p521	control vector	transfectants obtained
MCF7	p3v65	*RSU-1* vector	transfectants obtained
MCF10A	p1521	control vector	transfectants obtained
MCF10A	p3v36	*RSU-1* vector	transfectants obtained
MCF10A	p3v64	*RSU-1* vector	transfectants obtained
MCF10A	p521	control vector	transfectants obtained
MCF10A	p3v65	*RSU-1* vector	transfectants not obtained

It is interesting to note that the transfection of MCF10A cells with vector encoding *RSU*-1 cDNA under the control of a constitutive promoter did not result in the isolation of any G418 resistant colonies. It appears that the growth suppressive properties of Rsu-1 are sufficient to inhibit the growth of normal cells expressing this protein at high level. MCF10A clones derived from infection of cells with a retroviral vector containing Rsu-1 under the control of an inducible promoter have been isolated and are being screened for inducibility.

MCF 7 TRANSFECTANTS

Transfectants in the MCF 7 cell line are being analyzed for expression of *RSU-1* and the effect of *RSU-1* expression on signal transduction in the Ras pathway. The results shown below are those obtained from MCF 7 transfectants derived following transfection of p3v65 which encodes HA-tagged Rsu-1.

Expression of *RSU-1* RNA in MCF 7 cell clones (Figure 1). Total RNA from individual clones was analysed by Northern blotting using a probe specific for the mouse *RSU-1* ORF. This probe primarily detects the vector-encoded *RSU-1* , which was derived from the mouse *RSU-1* cDNA. At high stringency it hybridizes poorly with endogenous human *RSU-1*. The results indicated that most clones contained several *RSU-1* specific RNA species. RNA initiating in the RSV promoter and terminating at the poly A site contained in the *RSU-1* cDNA was expected to be about 1.7 kb in length. RNA initiating at the SV40 promoter and terminating at a vector encoded polA signal and site was predicted to 2.2kb in length. Several clones containing *RSU*- 1 specific RNA of these approximate sizes were

chosen for further study along with vector control clones and clones expressing larger size *RSU-1* specific transcripts. The clones chosen for greater study are those designated with an asteric *.

Expression of Rsu-1, Ras and Rac protein in MCF7 transfectant cell clones (Figure 2). Cell lysates were analyzed by Western blotting for the expression of p33 HA-Rsu-1 using a mouse monoclonal antibody directed against the HA epitope tag (clone 12CA5). Expression of p21 Ras was detected using a mouse monoclonal antibody which detects Ha-, Ki-, and N-Ras species (OP22). p21 Rac was also detected using a monoclonal antibody for Rac (Transduction Laboratories). The PVDF filter was reacted with the primary antibody which was detected using horse radish peroxidase-conjugated secondary antibody and ECL detection. The filter was then stripped and reprobed with each antibody sequencially. The results indicate that the HA-Rsu-1 was detected in all the transfectants except 3v65-2A, a clone which had predominantly a larger size RNA. In addition, the levels of HA-Rsu-1 were somewhat higher in two of the clones, 3v65-9B and 3v65-14B. The levels of Ras and Rac may be lower in these cell clones than in the controls. However, definitive information on the levels of Ras depend on additional experiments including 35S-labeling and immunoprecipitaion. Assays for the level and the function of RasGAP are underway in these clones.

The effect of Rsu-1 expression on the activation of the Ras signal transduction pathway (figures 3, 4, and 5). Treatment of cells with growth factor, serum or TPA was used to activate Ras; the activation of specific kinases which are dependent on specific Ras effectors was tested in control as well as HA-Rsu-1 expressing clones. The AKT kinase activation is dependent on the Ras effector phosphatidyl inositol-3 kinase. AKT kinase activity can be activated in serum starved cells by the addition of serum to the cultures. The activation of AKT was measured 30 minutes after the addition of serum by Western blotting of total cell protein initially with an antibody which reacts with the phosphorylated version of AKT kinase (New England Nuclear). This was followed by stripping and reprobing of the filter with an antibody which detects both phosphorylated and nonphosphorylated forms of AKT kinase. The results (figure 3) demonstrated that the HA-Rsu-1 expressing clones had a slighltly lower level of phosphorylated AKT kinase following serum starvation. Phosphorylation of AKT kinase was achieved in the clones in response to serum. These results suggest that the activation of phosphoinositol-3-kinase by Ras and the subsequent activation of AKT kinase was achieved and that Rsu-1 expression did not disrupt this pathway.

Next the activation of Erk2 was tested in serum starved HA-Rsu-1 clones and control cell lines. Cells were stimulated for 7.5 minutes with Epidermal Growth Factor (EGF) (100ng/ml) or for 10 minutes with TPA (100ng/ml). The cells were lysed and Erk2 was immunoprecipitated and assayed in an *in vitro* kinase assay using myelin basic protein (MBP) as a substrate [8]. MBP was separated by SDS-PAGE, transferred to filter, and exposed used for autoradiography (figure 4); in additon, the labeled MBP on the filter was quantitated (figure 5). In agreement with results obtained by us in other cell types there is an increase in the activation of Erk2 in response to EGF in all clones, and an increase in response to TPA in the clones which express the highest levels of HA-Rsu-1.

The results of the activation of Jun kinase by growth factor has been tested using a similar approach to that described above for the AKT. The results, not shown here, indicated that in serum starved MCF 7 cells there was constitutive phosphorylation of p46 jun kinase which did not appear to change with the addition of serum. However, there was an increase in the phosphorylation of p54Jun kinase in response to serum in the control cell lines which may be reduced in the HA-Rsu-1 transfectants. These experimental results are in agreement with those reported by us previously and and are being explored in more detail using *in vitro* kinase assays.

Biological Properties of MCF7 clones expressing HA-Rsu-1(figure 6). Anchorage dependent growth of MCF7 cells expressing HA-Rsu-1 was compared to wild type MCF7 cells and a vector control cell line. The results, in the form of a growth curve, demonstrate that while the plating efficiency of the clones was slightly lower than the control cell lines, the growth rates were similar. Anchorage

independent growth was also tested and the results indicated that some of the clones produced fewer colonies than the vector control transfectant and untransfected MCF 7 cell lines. One cell line, 3v65-14B-MCF7 exhibited a 90% reduction in colony formation compared to the control cel line. This cell line, 3v65-14B-MCF7, produces the highest level of HA-Rsu-1 protein.

Cell Line	Colony formation in soft agar*
MCF7	235 colonies, 2.3%
3v65-6B-MCF7	176 colonies, 1.75%
3v65-8B-MCF7	185 colonies, 1.85%
3v65-14B-MCF7	20 colonies, 0.2%

* 10,000 cells were plated per 60 mm dish in 0.3% agar. Colonies were counted microscopically 14 days post plating. The number is the average of two plates. The data is presented as the percentage of cell plated which formed colonies.

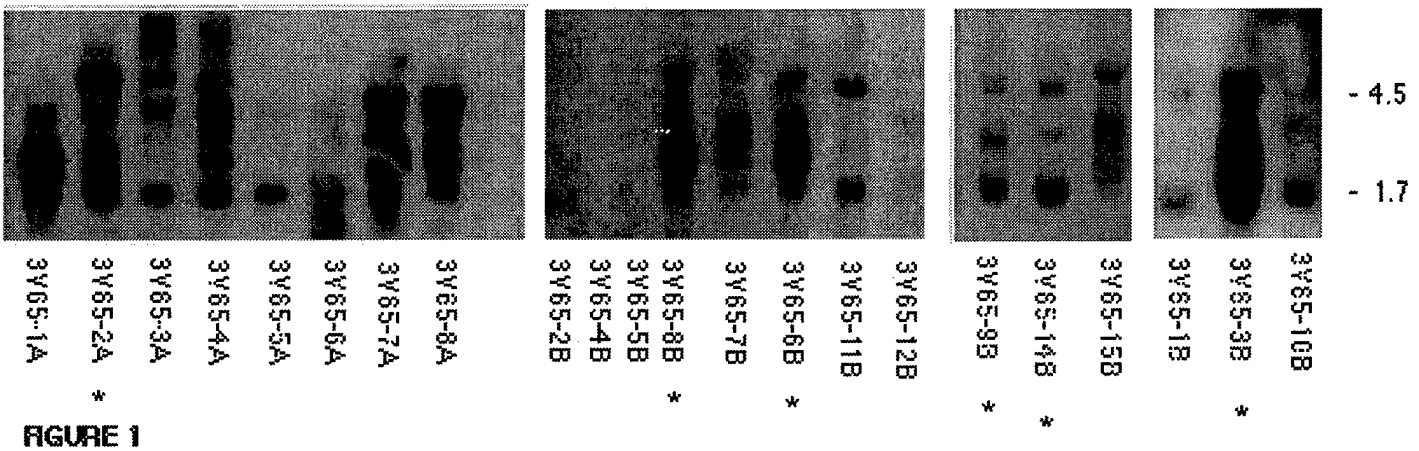
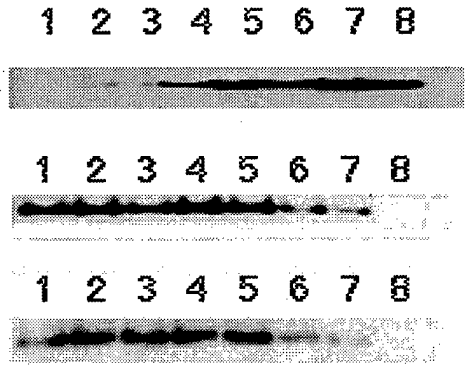


FIGURE 1



p33 RSU-1 (HA-TAG)

p21RAS

p21RAC

1= MCF7
2= 1529-1
3= 3V65-2A
4= 3V65-3B
5= 3V65-6B
6= 3V65-8B
7= 3V65-9B
8= 3V65-14B

FIGURE 2

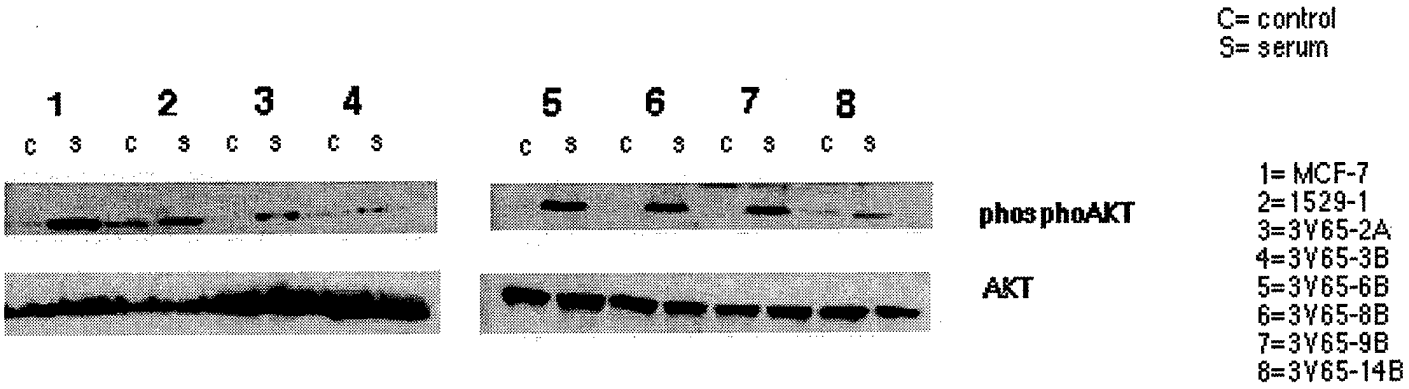


FIGURE 3

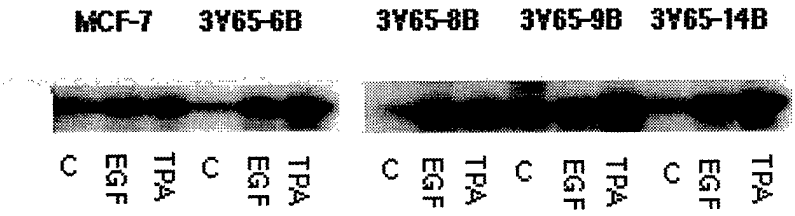
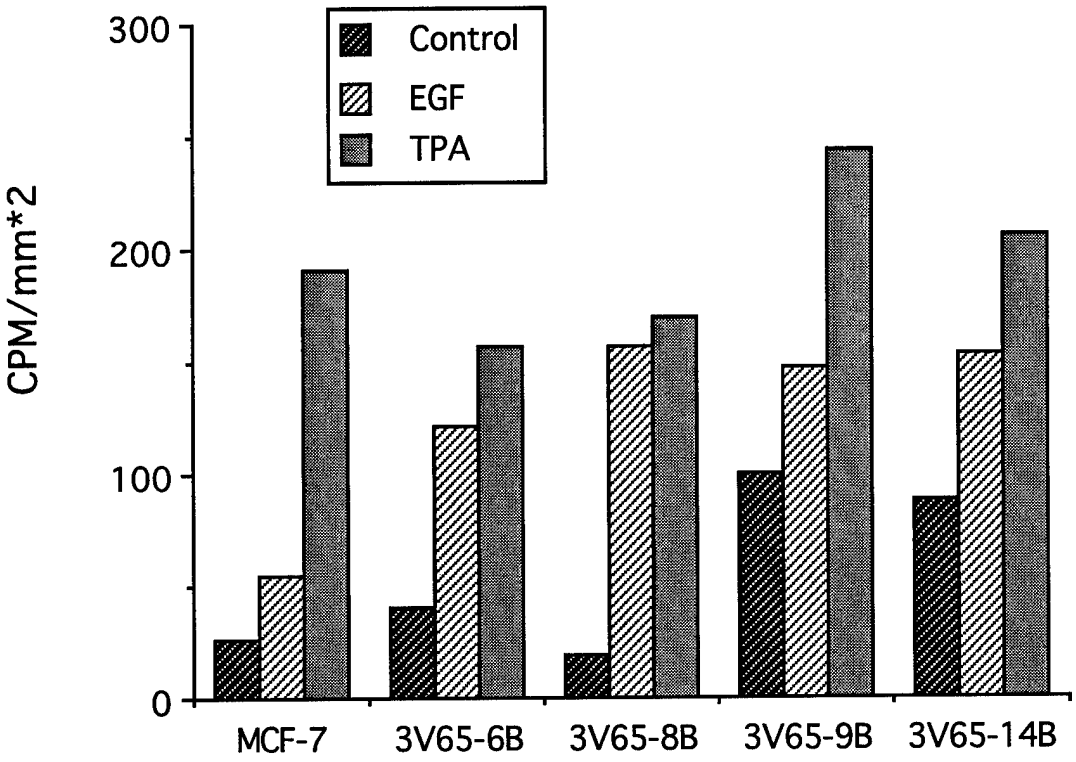


FIGURE 4

FIGURE 5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200
100
0
CPM/mm*2
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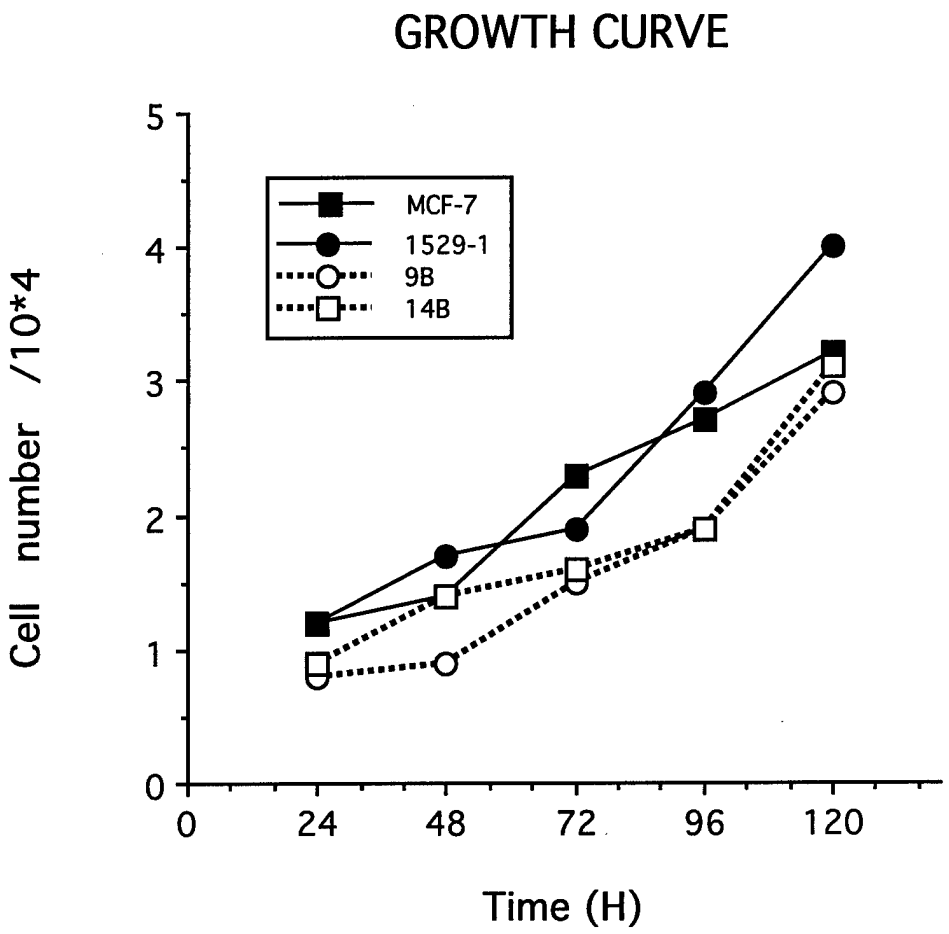
EGF
TPA
MCF-7
3V65-6B
3V65-8B
3V65-9B
3V65-14B

Figure 6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Cell number /10*4
Time (H)
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1529-1
9B
14B
0
1
2
3
4
5
0
24
48
72
96
120

CONCLUSIONS:

Rsu-1 transfectants have been successfully prepared in two cell lines, MCF7 and MCF10A. Analysis of the influence of Rsu-1 expression on Ras-dependent pathways has indicated that there is an increase in the activation of Erk 2 in response to EGF and TPA. There is a decrease in the activation of Jun kinase in response to serum, but no influence on the activation of AKT kinase by addition of serum to starved cells. The expression of Rsu-1 does not have a potent effect on the growth rate, but does appear to inhibit anchorage independent growth. The results reported here are preliminary in nature. As the experiments are repeated and the conditions refined for the cell lines and the conditions used, the differences between the Rsu-1 and control clones may be easier to detect and interpret. The results indicate that the expression of Rsu-1 does influence signal transduction "downstream" of Ras. These transfectants will allow the deliniation of the role of various Ras effectors in the activation of downstream kinases in human mammary carcinoma cell lines. Correlation of biochemical changes to inhibition of speciifc biological properties should point to speciifc signal transduction pathways responsible for speciifc biological effects. At present the role of Rsu-1 in altering signal transduction in the Ras pathway in response to growth factor and serum has been tested. The results indicate that specific pathways, controlled by specific Ras effectors, are altered. Further experimentation will allow determination of the effect of the number of EGF receptors, and hence the level of activation of the Ras pathway, on the influence of *RSU-1* on downstream kinase pathways. Also, the inluence of alteration of Ras pathway on estrogen stimulation of the cells can be determined.

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